This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS

- TEXT CUT OFF AT TOP, BOTTOM OR SIDES

- FADED TEXT

- ILLEGIBLE TEXT

- SKEWED/SLANTED IMAGES

- COLORED PHOTOS

- BLACK OR VERY BLACK AND WHITE DARK PHOTOS

- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

(51) International Patent Classification [7] : C12N 15/12, A61K 48/00, G01N 33/50, A01K 67/027, C12N 5/08	A2	(11) International Publication Number: WO 00/20582
		(43) International Publication Date: 13 April 2000 (13.04.00)

(21) International Application Number: PCT/US99/22364

(22) International Filing Date: 24 September 1999 (24.09.99)

(30) Priority Data:
09/164,664   1 October 1998 (01.10.98)   US

(71) Applicant: UNIVERSITY OF IOWA RESEARCH FOUNDA-TION [US/US]; Oakdale Research Campus, 100 Oakdale Campus #214 TIC, Iowa City, IA 52242–5000 (US).

(72) Inventors: CAMPBELL, Kevin, P.; 931 Evergreen Court, Iowa City, IA 52245 (US). HOLT, Kathleen, H.; 1601 Red Oak Drive, Corlville, IA 52241 (US). DUCLOS, Franck; P.O. Box 776, Washington Crossing, PA 18977 (US). LIM, Leland, E.; 478 Hawaii Court, Iowa City, IA 52246 (US). STRAUB, Volker; Bocklinstrasse 2, D–45147 Essen (DE). DAVIDSON, Beverly; 4004 El Paso Drive, Iowa City, IA 52246 (US). WILLIAMSON, Roger; 4235 Turkey Creek Road, Northeast, Iowa City, IA 52240 (US).

(74) Agent: FARRELL, Kevin, M.; P.O. Box 999, York Harbor, ME 03911 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: GENE REPLACEMENT THERAPY FOR MUSCULAR DYSTROPHY

(57) Abstract

Disclosed is a method for treating a patient suffering from the disease sarcoglycan–deficient limb–girdle muscular dystrophy by gene replacement therapy. Sarcoglycan gene replacement therapy produces extensive long–term expression of the sarcoglycan species which restores the entire sarcoglycan complex, results in the stable association of alpha–dystroglycan with the sarcolemma, and eliminates the morphological markers of limb–girdle muscular dystrophy. In another aspect, the invention relates to a method for determining a specific defective sarcoglycan species in the tissue of a patient. In another aspect, the invention relates to a mouse, and cells derived therefrom, homozygous for a disrupted alpha–sarcoglycan gene. In another aspect, the invention relates to methods for screening for therapeutic agents useful in the treatment of sarcoglycan–deficient limb–girdle muscular dystrophy.

GENE REPLACEMENT THERAPY FOR
MUSCULAR DYSTROPHY

Background of the Invention.

　　The term muscular dystrophy describes a group of
5　　diseases characterized by hereditary progressive muscle
weakness and degeneration. Several muscular dystrophies
are caused by mutations in genes that encode sarcolemmal
proteins, including certain types of limb-girdle muscular
dystrophy (LGMD). LGMD is genetically and clinically
10　　heterogeneous; it may be inherited in an autosomal
dominant or recessive manner, and may have different
rates of progression and severity. A unifying theme
among the LGMDs is the initial involvement of the
shoulder and pelvic girdle muscles, with relative sparing
15　　of most other muscle groups (Jackson et al., *Pediatrics*
41, 495-501 (1968); Bushby, K. M., *Neuromusc Disord* **5**,
71-74 (1995)).

　　The pace of discovery in the field of muscular
dystrophy research has been rapid since the discovery of
20　　the Duchenne and Becker muscular dystrophy (DMD) gene in
1986 (Monaco et al., *Nature* **323**, 646-650 (1986)). The
DMD gene encodes dystrophin, a large cytoskeletal protein
that together with other molecular components makes up
the dystrophin-glycoprotein complex (DGC). The
25　　dystrophin-glycoprotein complex (DGC) is a large
oligomeric complex of sarcolemmal proteins and
glycoproteins in skeletal and cardiac muscle (Campbell,
K.P. *Cell* **80**, 675-679 (1995); Ozawa et al., *Hum. Mol.
Genet.* **4**, 1711-1716 (1995)). This complex consists of
30　　dystrophin, a large cytoskeletal protein which binds F-
actin; α- and β-dystroglycan, which bind laminin and the
cysteine-rich region of dystrophin, respectively; α-, β-,
γ-, and δ-sarcoglycan (δ-SG), which form a distinct
subcomplex; and sarcospan, a 25 kDa protein predicted to
35　　span the membrane four times (Crosbie et al., *J. Biol.
Chem.* **272**, 31221-31224 (1997). The DGC spans the
sarcolemma and is believed to play an essential role in
maintaining the normal architecture of the muscle

sarcolemma by constituting a link between the
subsarcolemmal cytoskeleton and the extracellular matrix.
This structural linkage is thought to protect muscle
fibers from the mechanical stress of contraction.

5   Mutations in different components of the DGC lead to
similar dystrophic features, suggesting that the function
of the DGC as a whole is dependent on intact molecular
interactions between its individual subunits. The loss
of one component destroys the link, and leads to muscle
10 fiber degeneration. Several components of the DGC have
been implicated in several human muscular dystrophies
(Straub et al., *Curr. Opin. Neurol.* 10, 168-175 (1997)).
Mutations in dystrophin cause Duchenne and Becker
muscular dystrophy (DMD) (Hoffman et al., *Cell* 51, 919-
15 928 (1987)). Two forms of congenital muscular dystrophy
are caused by mutations in the extracellular matrix
protein laminin 2 (Helbling-Leclerc et al., *Nature Genet.*
11, 216-218 (1995); Allamand et al., *Hum. Mol. Genet.* 6,
747-752 (1997)). Mutations in each of α-, β-, γ-, and δ-
20 SG cause autosomal recessive LGMD types 2D, 2E, 2C, and
2F, respectively (Roberds et al., *Cell* 78, 625-633
(1994); Piccolo et al., *Nature Genet.* 10, 243-245 (1995);
Lim et al., *Nature Genet.* 11, 257-265 (1995); Bönneman et
al., *Nature Genet.* 11, 266-273 (1995); Noguchi et al.,
25 *Science* 270, 819-822 (1995); Passos-Bueno et al., *Hum.*
Mol. Genet. 5, 815-820 (1996); Nigro et al., *Hum. Mol.*
Genet. 5, 1179-1186 (1996); Nigro et al., *Nature Genet.*
14, 195-198 (1996)).

        In the sarcoglycan-deficient LGMDs, a mutation
30 effecting the function of a particular sarcoglycan
species leads to the loss or dramatic reduction of all
four sarcoglycan proteins from the sarcolemma. Four
types of LGMD, LGMD2D, LGMD2E, LGMD2C and LGMD2F, are
known to be caused by mutations in distinct sarcoglycan
35 genes, α-, β-, γ- and δ-SG respectively (Roberds et al.,
Cell 78, 625-633 (1994); Piccolo et al., *Nature Genet.*
10, 243-245 (1995); Lim et al., *Nature Genet.* 11, 257-265

-3-

(1995); Bönneman et al., *Nature Genet.* **11**, 266-273
(1995); Noguchi et al., *Science* **270**, 819-822 (1995);
Passos-Bueno et al., *Hum. Mol. Genet.* **5**, 815-820 (1996);
Nigro et al., *Hum. Mol. Genet.* **5**, 1179-1186 (1996); Nigro
5   et al., *Nature Genet.* **14**, 195-198 (1996)).
Unfortunately, functional characterization of the
sarcoglycan complex has proven elusive.

The BIO 14.6 hamster is a widely studied animal
model of muscular dystrophy and cardiomyopathy (Homburger
10   et al., 1962). In this animal, the sarcoglycan complex
is missing and dystrophic features, including central
nucleation and necrosis of muscle fibers, are evident.
Previous studies have documented a disruption in the
integrity of the DGC in skeletal and cardiac muscle of
15   this animal, with a dramatic decrease in the amount of α-
dystroglycan (Roberds et al., *J Biol Chem.* **268**, 11496-
11499 (1993), Iwata et al., *FEBS* **329**, 227-231 (1993)).
Recently, it was shown that a mutation in the δ-SG gene
of BIO 14.6 hamsters leads to sarcoglycan complex
20   disruption and dystrophic changes (Okazaki et al., *Nature
Genet.* **13**, 87-90 (1996); Nigro et al., *Hum. Mol. Genet.*
6, 601-607 (1997); Sakamoto et al., *Proc. Natl. Acad.
Sci.* **94**, 13873-13878 (1997)). These findings make the
animal a useful model for the study of human sarcoglycan-
25   deficient LGMD.

While the BIO 14.6 hamster model is useful for
certain studies, the generation of additional models of
sarcoglycan-deficient LGMD would greatly facilitate
progress towards understanding of the disease. The use
30   of animal models deficient in the various sarcoglycan
species to study the effects of these specific
deficiencies, would help identify the exact role of each
species in the DGC. Additionally, since there are no
known cases of a LGMD from a primary sarcoglycan defect,
35   with concomitant muscular dystrophy and cardiomyopathy,
the generation of a phenotypically more accurate model of
AR-LGMD would be beneficial to the research effort.

-4-

Summary of the Invention.

In one aspect the present invention relates to a method for treating a patient with the disease sarcoglycan-deficient limb-girdle muscular dystrophy by gene replacement therapy. Sarcoglycan gene replacement therapy produces extensive long-term expression of the deficient sarcoglycan species which restores the entire sarcoglycan complex, results in the stable association of alpha-dystroglycan with the sarcolemma, and eliminates the morphological markers of limb-girdle muscular dystrophy.

In another embodiment, the invention relates to a method for determining a specific defective sarcoglycan species in the tissue of a patient. The method involves culture of muscle cells obtained from the patient, and the independent introduction of expression vectors encoding each of the sarcoglycan species, α, β, γ, and δ, into the cultured cells, with subsequent assaying for restoration of the dystrophin-glycoprotein complex.

In another embodiment, the invention relates to a mouse, and cells derived therefrom, that is homozygous for a disrupted α-sarcoglycan gene. The disruption prevents the synthesis of functional α-sarcoglycan in cells of the mouse and results in the mutant mouse having no detectable sarcospan, β-, γ-, δ-sarcoglycan, and having reduced α-dystroglycan in the sarcolemma of skeletal and cardiac muscles, as well as a reduction of dystrophin in skeletal muscle, when compared to tissue of a mouse lacking a disrupted α-sarcoglycan gene.

In another embodiment, the invention relates to methods for screening for therapeutic agents useful in the treatment of sarcoglycan-deficient limb-girdle muscular dystrophy. The methods involve administering a candidate therapeutic agent to a mouse, or cells derived therefrom, and assaying for therapeutic effects on the mouse or cells, with therapeutic effects being a

-5-

reduction or reversal in disease progression, or a restoration of the dystroglycan complex.

Brief Description of the Drawings.

Figure 1 is a schematic of the human sarcoglycan
5   proteins. Putative transmembrane domains (TM), cysteine clusters (cysteines) and asparagine residues within glycosylation acceptor motifs (N) are located as indicated. α-SG is a type I transmembrane glycoprotein with a signal sequence (signal) at the amino-terminus.
10 β-, γ-, and δ-SG are type II transmembrane glycoproteins with intracellular amino-termini and extracellular carboxyl-termini. δ^Δ-SG is truncated at the carboxyl-terminus to mimic a mutation identified for LGMD2F.

Figure 2 is a diagrammatic representation of data
15 from experiments measuring the percent of myofibers containing centrally located nuclei from F1B controls, BIO 14.6 hamsters, and BIO 14.6 hamsters with persistent expression of the δ-SG adenovirus. Sections from F1B, BIO 14.6 and BIO 14.6 hamsters injected with δ-SG
20 adenovirus were examined and scored. In the case of BIO 14.6 hamsters injected with δ-SG adenovirus, only fibers converted to sarcoglycan-positive were counted. The number of fibers examined for each sample is given in parentheses and the s.e.m. is shown.

25   Figure 3 is a restriction map of the wild-type Sgca locus (Sgca⁺), the targeting construct, and the targeted locus. A region of 902 bp including exons 2 and 3 (E2, E3) was deleted and replaced by a PGK-neomycin cassette (NEO). Generation of Sgca-null mutant mice. The probe
30 used in Southern blot analysis is depicted by the black box. Primers for PCR analysis are depicted by horizontal arrows.

Figure 4 is a diagrammatic representation of the activity of muscle specific pyruvate kinase (PK) in 7 to
35 10 week old wild type (+/+), heterozygotes (+/-), homozygotes (-/-), and mdx mice. Error bars indicate the

-6-

standard deviation where n equals the number of mice in each set.

Figure 5 is a diagrammatic representation of data on body mass, muscle mass, absolute maximum isometric
5 tetanic force, specific force, and peak force during resistance to stretch of passive muscles for the different mice assayed. The data on EDL and soleus muscles of the *Sgca*-null mutant mice are represented as a percentage of the values for muscles of control mice.
10 Significant differences between the data obtained in *Sgca*-deficient and control mice are indicated by an asterisk. All data are presented as the mean ± one standard error of the mean.

Detailed Description of the Invention.
15   The present invention is based on the discovery that sarcoglycan gene replacement can correct primary mutations in individual sarcoglycan components *in vivo* and lead to the restoration of the DGC. More specifically, experiments in the Exemplification section
20 which follows demonstrate therapeutic delivery of a deficient sarcoglycan gene to produce extensive long-term expression of the sarcoglycan species in animal models. The restored expression of the deficient species rescues the entire sacoglycan complex. This restoration of the
25 sarcoglycan complex results in stable association of alpha-dystroglycan with the sarcolemma and eliminates the morphological markers of limb girdle muscular dystrophy.

One embodiment of the present invention is a method for the treatment of a patient with sarcoglycan-deficient
30 LGMD, caused by a mutation in one of the sarcoglycan species, α, β, γ, or δ, through the expression of the deficient species in the affected tissue from an exogenous source of genetic material encoding the sarcoglycan protein. The deficient sarcoglycan species
35 is determined, and then corrected by introduction of a therapeutic amount of genetic material encoding a

-7-

functional version of the deficient species, to the affected tissue, under conditions appropriate for expression. The specific sarcoglycan deficiency can be diagnosed by several methods and can be performed to
5   further diagnose a symptomatic individual, or to screen presymptomatic individuals. Such screening includes prenatal screening.

Several mutations in the various sarcoglycan genes have been identified as contributing to LGMD, and others
10 are likely to be identified in the future (Piccolo et al., *Nat. Genet.* **10**, 243-245 (1995); Piccolo et al., *J Med Genet.* **34**, 470-475 (1997); Duggan, et al., *Neurogenetics* **1**, 827-834 (1997); Duggan et al., *N Engl J Med.* **336**, 618-624 (1997); Carrier et al., *J Med Genet.*
15 **34**, 470-475 (1997)). Mutations that cause a change in expression levels or a change in protein size, such as mutations in transcription or translation regulatory elements, destabilizing point mutations, frame shift, or missense mutations, can be detected by examination of the
20 sarcoglycan proteins themselves via immunodiagnostics of a muscle tissue sample using α, β, γ, or δ-SG specific antibodies (Campbell et al., (1995) U.S. Patent 5,418,139; Campbell et al., (1994) U.S. Patent 5,308,752; Campbell et al., (1997) U.S. Patent 5,672,694), and
25 comparison to wild-type. Alternatively, the genetic material encoding the individual SG genes of a patient can be examined for significant mutations by several different techniques. cDNA or mRNA specific for the individual SG genes can be examined quantitatively for
30 reduction in transcript levels, and also for detectable size differences indicative of frame shift or missense mutations by the techniques of DNA or RNA hybridization analysis, or by amplification using the polymerase chain reaction (PCR).
35 Mutations can also be identified through sequence analysis of genetic material, by either direct sequencing or probing for specific mutations. Hybridization

-8-

analysis using nucleic acid probes can identify specific point mutations in a given sarcoglycan gene. Such diagnostic methods are performed by isolating nucleic acids, either genomic DNA, cDNA or mRNA, from an
5 individual and testing the diagnostic probes for the ability to hybridize to the isolated nucleic acid. When necessary, the nucleic acid sequence can be amplified by the PCR prior to hybridization analysis. A working example of this technique uses specific oligonucleotides
10 designed to hybridize to specific mutations in the β-SG gene (Campbell et al., (1997) U.S. Patent 5,672,694). As more mutations in the SG genes are identified as contributing to LGMD, similar oligo probes can be designed and used for rapid diagnostic analysis. In lieu
15 of the development of specific oligo probes, mutations can be identified by sequence analysis of individual SG genes. The genetic material of the individual SG genes can be sequenced, and mutations that would adversely effect expression or protein structure can be identified
20 by comparison to wild type sequences (Campbell et al., (1997) U.S. Patent 5,672,694).

Alternatively, the functional absence of a particular sarcoglycan species can be diagnosed through functional analysis of the cellular complement of
25 sarcoglycan proteins. One example of such an assay is described in detail below.

Once a specific sarcoglycan deficiency is established, a functional replacement gene can be introduced to the effected tissue of the patient, under
30 conditions appropriate for expression. This can be accomplished through the use of an expression vector to deliver sequences that encode a functional SG protein. An appropriate expression vector can safely and efficiently deliver exogenous nucleic acid to a recipient
35 cell in the patient. In order to achieve effective gene therapy, the expression vector used must be designed for efficient cell uptake and gene product expression, e.g.

-9-

an adenovirus based or adeno-associated virus (AAV) based
gene delivery vector (Berkner, K.L., *Current Topics in
Microbiol. and Imuñol.* 158, 39-66 (1992); Stratford-
Perricaudet et al., *Hum. Gene Ther.* 1, 241-256 (1990);
5 Rosenfeld et al. *Cell* 8, 143-144 (1992); Stratford-
Perricaudet et al. *J. Clin. Invest.* 90, 626-630 (1992)).
Specific methods of application of the present invention
depend largely upon the expression system employed,
however, most involve insertion of sarcoglycan coding
10 sequence at an appropriate position within the expression
vector, and subsequent delivery of the expression vector
to the target tissue for expression.

In the preferred embodiment, a nucleotide sequence
encoding the deficient sarcoglycan gene is inserted into
15 an adenovirus based expression vector. Several genes
have been successfully expressed using adenovirus based
vectors including p53 (Wills et al., *Human Gene Therapy*
5, 1079-188 (1994), dystrophin (Vincent et al., *Nature
Genetics* 5, 130-134 (1993), erythropoietin (Descamps et
20 al., *Human Gene Therapy* 5, 979-985 (1994), ornithine
transcarbamylase (Stratford-Perricaudet et al., *Hum. Gene
Ther.* 1, 241-256 (1990), adenosine deaminase (Mitani et
al., *Human Gene Therapy* 5, 941-948 (1994), interleukin-2
(Haddada et al., *Human Gene Therapy* 4, 703-711 (1993),
25 and α-1-antitrypsin (Jaffe et al., *Nature Genetics* 1,
372-378 (1992). The use of adenovirus based vectors in
gene therapy is considered promising for a number of
reasons, including the wide range of host cells, and the
mechanism of expression from the adenoviral vector which
30 occurs without chromosomal integration, eliminating the
risk of insertional mutagenesis. Gregory et al., (1997)
U.S. Patent 5,670,488; McClelland et al., (1998) U.S.
Patent 5,756,086; Armentano et al., (1998) U.S. Patent
5,707,618; Saito et al., (1998) U.S. Patent 5,731,172,
35 herein incorporated by reference, describe several
recently developed adenovirus based expression vectors,
and their use in gene therapy. Alternatively, a gutted

-10-

adenovirus delivery system can be used (Clemens et al.,
Gene Therapy 3, 965-972 (1996)).

An adeno-associated virus (AAV) based vector can
also be used as a delivery system. Some examples are
5   described by Carter et al., (1989) U.S. Patent 4,797,368;
Lebkowski et al., (1992) U.S. Patent 5,153,414;
Srivastava et al., (1993) U.S. Patent 5,252,479;
Lebkowski et al., (1994) U.S. Patent 5,354,678; Wilson et
al., (1998) U.S. Patent 5,756,283, herein incorporated by
10   reference. AAV is an integrating DNA parvovirus, a
naturally occurring defective virus that requires other
viruses, such as adenovirus or herpes viruses as helper
viruses ("Handbook of Parvoviruses", ed., P. Tijsser, CRC
Press, (1990)). AAV vectors have been demonstrated
15   functional in a wide variety of cell types, including
differentiated and non-dividing cells, suggesting a
potential for this vector system for successful *in vivo*
gene delivery to muscle tissue.

Other possible gene expression systems for
20   sarcoglycan gene therapy include retroviral based vectors
and delivery systems (Miller et al., *Blood* 76, 271
(1990), and also plasmid based nucleic acid delivery
systems (Eastman et al., (1998) U.S. Patent 5,763,270).

In the preferred embodiment, a cytomegalovirus
25   promoter element is used to drive gene expression from
the expression vector. However, the expression vector
can utilize a variety of regulatory sequences to achieve
a therapeutic level of expression. Tissue specific
regulatory sequences can also be used to restrict gene
30   expression to a specific target tissue (Kuang et al., *J.
Clin. Invest.* 102, 844-852 (1998)).

The method of delivery of the gene expression system
to the target tissue varies with the expression system
used. In preferred embodiments, upon diagnosis of the
35   deficient sarcoglycan species, an appropriately packaged
adenovirus based expression vector construct is delivered
via intramuscular injection of the sarcoglycan-deficient
muscle tissue. Recipient tissue comprises muscle in the

patient that is, or is predicted to be, affected by the
sarcoglycan gene deficiency. Administration of the gene
to all deficient muscle tissues represents one
therapeutic option. However, significant therapeutic
5   benefits can also be achieved by selective administration
to specific target muscles, to restore or prevent the
loss of specific motor functions. For example, specific
treatment of a small number of muscle groups can restore
or prevent further deterioration of function, thereby
10   enabling the patient to continue to eat without
assistance.

        Administration of the deficient sarcoglycan gene
should optimally occur at as early a stage in disease
progression as diagnosis permits, preferably, prior to
15   the onset of severe muscle damage. Genetic diagnosis of
the disease prior to the onset of the pathology allows
gene therapy intervention at an extremely early stage in
life. Evidence presented in the Exemplification
indicates that significant therapeutic benefits may be
20   obtained from such early intervention.

        The present invention also provides methods for
diagnosing a specific sarcoglycan deficiency of a patient
suspected of suffering from sarcoglycan-deficient LGMD.
The method is based on the observation that restoration
25   of a single deficient sarcoglycan species can restore the
entire sarcoglycan complex and lead to the complete
restoration of the DGC. In the preferred embodiment,
cells from a biopsy of muscle tissue are propagated in
culture under conditions appropriate for the formation of
30   myotubes. An expression vector that encodes one of the
sarcoglycan species is introduced to the muscle cells,
and the recipient cells are then examined, through either
morphological or biochemical assays, for restoration of
their dystrophin-glycoprotein complex. Delivery of the
35   deficient sarcoglycan species should produce a functional
DGC.

        As detailed in the Exemplification section, delivery
of a recombinant adenovirus vector expressing δ-SG

-12-

restores the entire sarcoglycan complex to the naturally
δ-SG deficient sarcolemma in BIO 14.6 skeletal muscle,
restoring the integrity of the sarcolemma. This
restoration produces a coincident stabilization of α-

5   dystroglycan, the extracellular laminin-binding component
of the DGC and eliminates characteristic features of
disease progression in recipient myofibers.
Additionally, injection of a recombinant adenovirus
expressing α-sarcoglycan into muscle of a mouse with a

10   homozygous α-SG gene deficiency restores functional
sarcoglycan complex to the sarcolemma, further
demonstrating the feasibility of gene transfer for the
different species of sarcoglycan-deficient-LGMD. These
experiments confirm the feasibility of gene delivery

15   therapy to treat sarcoglycan-deficient LGMD. This
disclosure constitutes the first example of sarcoglycan
gene replacement and demonstrates that primary mutations
in individual sarcoglycan components can be corrected in
vivo.

20   Another aspect of the current invention relates to
the development of mice with homozygous null mutations in
specific sarcoglycan genes that develop a LGMD phenotype.
Evidence indicates that a defect in one of either, α-, β-
, γ-, or δ-sarcoglycan genes causes sarcoglycan-deficient

25   LGMD (Roberds et al., Cell 78, 625-633 (1994); Piccolo et
al., Nature Genet. 10, 243-245 (1995); Lim et al., Nature
Genet. 11, 257-265 (1995); Bönneman et al., Nature Genet.
11, 266-273 (1995); Noguchi et al., Science 270, 819-822
(1995); Passos-Bueno et al., Hum. Mol. Genet. 5, 815-820

30   (1996); Nigro et al., Hum. Mol. Genet. 5, 1179-1186
(1996); Nigro et al., Nature Genet. 14, 195-198 (1996);
Carrie et al., J Med Genetics 34, 470-475 (1997)).

            One embodiment of the present invention is a mouse,
or a cell derived therefrom, that is homozygous for a

35   disrupted α-SG gene. The Exemplification outlines the
production of a mouse line homozygous for a disrupted α-
SG gene, by targeted inactivation of an α-SG allele of an

-13-

embryonic stem cell, with subsequent implantation into
pseudopregnant females to generate heterozygous null
progeny. The resulting heterozygous progeny are then
subsequently bred to produce homozygous null mutants.
5   This disclosure presents the first engineered animal
models for autosomal recessive muscular dystrophy with a
primary sarcoglycan deficiency.

       Characterization of the resulting α-SG deficient
mice (α-SG-/-) indicates that they develop typical
10 histological features of muscular dystrophy including
central nucleation, myofibrosis, and necrosis of muscle
fibers, and also loss of the entire sarcoglycan complex
along with the reduction of α-dystroglycan, sarcospan,
and dystrophin from the sarcolemma. Biochemical evidence
15 of the tight association of the sarcoglycan proteins with
each other has been previously reported (Ervasti et al.,
Cell **66**, 1121-1131 (1991); Yoshida et al., *Eur J Biochem*
222, 1055-1061 (1994); Jung et al., *FEBS* **381**, 15-20
(1996)). When taken together, these results indicate
20 that defects in any individual sarcoglycan species which
alter primary structure or spatio-temporal synthesis will
manifest as defects of the entire sarcoglycan complex,
producing a similar phenotype.

       α-SG-/- mice have a variety of uses as a murine
25 model system. One such use lies in the identification of
therapeutic agents for the treatment of LGMD. To
identify therapeutic genes or compounds, candidate agents
are delivered to the diseased tissue of the mouse, which
is then assayed for reversal of disease pathology, e.g.
30 the elimination of morphological markers for LGMD,
restoration of the DGC, restoration of the sarcoglycan
complex. Cells isolated from a α-SG-/- mouse can be
propagated in culture and used in a similar assay to
identify therapeutic agents. SG null mice, or cells
35 derived therefrom, can also be used experimentally to
identify functionally significant regions of the
deficient sarcoglycan molecules. Such assays involve the

-14-

re-introduction of the deficient sarcoglycan species, or
a portion thereof, with subsequent diagnosis of the
treated tissue or cells for restoration of wild type
function.

5   Exemplification

Injection of δ-SG plasmid DNA rescues the sarcoglycan
complex.

To test the hypothesis that δ-SG gene transfer could
restore the sarcoglycan complex in the BIO 14.6 hamster,
10   direct plasmid DNA injections into BIO 14.6 skeletal
muscle were performed. Previous studies have shown that
de novo expression of dystrophin in a small percentage of
fibers can be achieved by direct injection of plasmid DNA
expression vectors into mdx mouse skeletal muscle.
15   Expression of dystrophin in these experiments leads to
the restoration of the entire dystrophin-glycoprotein
complex. This methodology was adapted for the BIO 14.6
hamster in order to test the hypothesis that introduction
of normal copies of δ-SG DNA would lead to ordered
20   sarcoglycan complex assembly. Due to the overall
structural similarity of the sarcoglycan proteins to each
other, each of the individual sarcoglycans was tested in
this in vivo reconstitution assay.

Expression vectors were engineered to encode myc
25   epitope-tagged versions of human α-, β-,γ- and δ-SG, as
well as an extracellular deletion construct (δ^Δ-SG) which
recapitulates a mutation found in LGMD2F (Nigro et al.,
Nature Genet. 14, 195-198 (1996)). A schematic
representation of these proteins is shown in Fig. 1. The
30   sarcoglycans share a similar topology, with a single
putative transmembrane domain, a short intracellular
domain and a larger extracellular domain. The
extracellular domains contain clusters of cysteine
residues, which may form intra- and intermolecular
35   disulfide bonds, as well as consensus sequence

-15-

recognition sites for asparagine-linked glycosylation.
These constructs were transiently expressed in Chinese
hamster ovary (CHO) cells to examine the integrity of the
expressed proteins. Anti-myc immunoblot analysis of cell
5   lysates reveals that α-, β-, γ-, δ- and δ^Δ-SG expression
vector constructs direct synthesis of proteins which
fractionate by SDS-PAGE according to their predicted
molecular weights. As a control, cell lysate from mock-
transfected cells was shown to demonstrate specificity of
10   the antibody for the tagged proteins.

All sarcoglycan expression constructs were co-injected
with a β-galactosidase reporter construct to identify
fibers that harbored the DNA constructs. Expression of
δ-SG restored the sarcoglycan complex to the sarcolemma
15   based on sarcoglycan-specific antibody detection by
immunofluorescence of serial cryosections. In all δ-SG
cDNA-injected animals, expression of β-galactosidase in
muscle fibers was coincident with sarcoglycan expression.
In contrast, fibers expressing α-, β-, γ- and δ^Δ-SG did
20   not have detectable sarcoglycan proteins at the
sarcolemma, as determined by immunofluorscence. It is of
interest to note that even over-expression of γ-SG, which
is highly related at the amino acid sequence level
(approximately 60% identity) to δ-SG, cannot compensate
25   for the loss of δ-SG. Furthermore, deletion of the
extracellular cysteine-rich region of δ-SG, analogous to
one mutation identified for LGMD2F (δ^Δ-SG), blocked the
ability of δ-SG to rescue the sarcoglycan complex.
Although this technique is limited because of the low
30   efficiency of gene transfer (typically 1-2% of the fibers
take up and express the plasmid), the data suggest that
δ-SG is required for sarcoglycan complex assembly in the
BIO 14.6 hamster. Finally, the results indicate that key
structural determinants of δ-SG likely reside in the
35   extracellular cysteine-rich region of the protein.

Recombinant δ-SG adenovirus-mediated gene transfer into
the quadriceps femoris muscle of BIO 14.6 hamster.

    In order to achieve high efficiency of gene
transfer, an adenovirus construct encoding human δ-SG was
5 prepared for injection into hamster skeletal muscle. In
the δ-SG adenovirus-injected BIO 14.6 muscle, the
majority of the fibers were converted to δ-SG-positive.
Immunofluorescence of skeletal muscle sections, using an
antibody specific for δ-SG showed the extent of δ-SG
10 localized to the sarcolemma was similar to that of
unaffected control F1B hamster. In addition some
cytoplasmic staining was evident, possibly due to high
levels of expression generated by the CMV promoter.
Renewed expression of δ-SG at the sarcolemma was
15 coincident with the rescue of the entire sarcoglycan
complex to the sarcolemma based on α-, β- and γ-SG-
specific immunofluorescence of serial sections. In
addition, α-DG was restored to the sarcolemma in fibers
expressing δ-SG . 80% or more of the fibers were
20 converted to sarcoglycan-positive, as observed by low
power field examination of immunostained muscle sections.
To assess the persistence of δ-SG expression over time,
hamsters were injected with the δ-SG adenovirus and
examined at various time points with immunofluorescence.
25 Remarkably, δ-SG expression was evident at all time
points up to 9 months post-injection, without the use of
immunosuppressive agents.

    δ-SG expression resulting from hamstring injection
at 11 days was compared to δ-SG expression from injection
30 at 3-weeks.   Because of the small size of the 11 day old
muscle at injection and its tremendous growth over the
test period, similar levels of expression as in the 3-
week-old injected hamsters were not seen in the 11 day
old injected hamstrings. Nevertheless, long-term
35 expression of δ-SG following a single injection of the
adenovirus was achieved with both injection ages.

These results confirm the ability of δ-SG to restore
the entire sarcoglycan complex to the sarcolemma in the
BIO 14.6 hamster. An adenovirus construct encoding human
α-SG was also analyzed as a control. In uninjected BIO
5   14.6 quadriceps muscle and in BIO 14.6 quadriceps muscle
injected with the α-SG adenovirus, δ-SG was not detected
by immunofluorescence. Likewise, α-, β- and γ-SG
proteins were not present in serial sections.

Correction of the structural defects in the DGC following
10   rescue of the sarcoglycan complex in the BIO 14.6
hamster.
Biochemical studies were performed to determine if
the restored sarcoglycan complex associated correctly
with other components of the DGC. Glycoprotein
15   preparations from quadriceps muscle of F1B, BIO 14.6, and
BIO 14,6 injected with 10^9 particles of δ-SG adenovirus,
were examined by immunoblot analysis using antibodies
raised against δ-SG, the DGC and the 156 kDa α-
dystroglycan. BIO 14.6 muscle had no detectable δ-SG and
20   was found to have severe deficits in α-, β- and γ-SG as
well as α- dystroglycan, as shown previously. After a 14
day expression period, the BIO 14.6 muscle injected with
the δ-SG adenovirus exhibited renewed expression of δ-SG,
the entire sarcoglycan complex, and α-dystroglycan, all
25   at levels comparable to that of the control F1B. Equal
loading of the lanes was demonstrated by comparable
amounts of a non-DGC glycoprotein, the 140 kDa α_2 subunit
of the DHPR, and silver staining.
The reconstituted DGC was further characterized by
30   sucrose gradient sedimentation. Immunoblot analysis of
the fractions, using antibody directed against either α-
DG or the respective sarcoglycans, showed that α-
dystroglycan from the F1B muscle sedimented as two peaks,
and the sarcoglycans co-sedimented with α-dystroglycan in
35   the later peak fractions. In contrast, α-dystroglycan
from the BIO 14.6 muscle sedimented as a single peak and

-18-

no sarcoglycans were present, as expected. The earlier
sedimenting complex is thought to reflect a species of
dystroglycan that does not associate with the
sarcoglycans, possibly the dystroglycan at the
5   myotendinous and/or neuromuscular junctions. Western
blot analysis of the fractions also identified the
presence of δ-, α-, β-, and γ-SG using antibodies
specific for the respective proteins. This indicates
that expression of δ-SG in the BIO 14.6 muscle not only
10   restored the sarcoglycan complex to the sarcolemma, but
also re-established the association between the
sarcoglycan complex and α-dystroglycan as evidenced by
the co-fractionation of these proteins. Together, these
results provide strong evidence that the integrity of the
15   DGC has been restored in the BIO 14.6 muscle expressing
the δ-SG adenovirus.

Dystrophic features are greatly reduced in muscle
expressing δ-SG.

       Histological features of the dystrophic muscle in
20   the BIO 14.6 hamster include cellular infiltration,
variation in myofiber size and central nucleation.
Central nucleation is assumed to be a sign of continued
cycles of degeneration and regeneration, with the
earliest onset at 2 to 3 weeks of age in the BIO 14.6
25   hamster. However, genetic intervention at age three
weeks resulted in a marked reduction of the number of
centrally located nuclei in the injected BIO 14.6 hamster
compared to uninjected BIO 14.6 littermates at 9 weeks
post-injection, as determined by haematoxylon and eosin
30   staining and immunofluorescence. The percentage of
fibers with centrally-located nuclei was calculated from
multiple fields of age-matched samples of F1B (1.4%), BIO
14.6 (94%) and BIO 14.6 hamsters injected with the δ-SG
adenovirus (7.2%) (Fig. 2). Clearly, restoration of the
35   sarcoglycan complex significantly affects the

degenerative process in the δ-SG adenovirus-injected
muscle.

Sarcolemmal membrane integrity is normal in muscle fibers
expressing δ-SG.

5   An intact plasma membrane is a critical component of
the highly regulated transfer of molecules into and out
of muscle fibers. The stresses of contraction can induce
tears in the sarcolemma, allowing for release of muscle
enzymes and growth factors as well as the influx of

10   calcium. These transient breaks are thought to be one
mechanism by which skeletal muscle can sense and respond
to incremental changes in external load. In the case of
dystrophic muscle, chronic membrane disruptions are
thought to contribute to fiber necrosis. Evidence from

15   muscular dystrophies that arise from defects in the DGC
clearly demonstrates the importance of intact molecular
links between the cytoskeleton and the extracellular
matrix. Perturbation of this linkage with subsequent
loss of membrane integrity is an important pathogenic

20   mechanism leading to muscle cell necrosis.
           Sarcolemmal membrane permeability in live animals
was assessed to evaluate its potential role in the
pathogenesis of the BIO 14.6 hamster. It has been
demonstrated that Evans blue dye (EBD) can be used as a

25   tracer molecule to monitor sarcolemmal membrane integrity
in various dystrophic mouse models involving the absence
of dystrophin and/or utrophin. In the present study, EBD
was injected intravenously into 4 month old F1B, BIO 14.6
and BIO 14.6 hamsters transfected with the δ-SG

30   adenovirus, and dye uptake into muscle fibers was
examined after 72 hours. Macroscopically, EBD
incorporation into skeletal muscle was clearly visible in
various muscle groups in all BIO 14.6 hamsters, whereas
no dye incorporation into muscle could be detected in the

35   F1B hamsters. The quadriceps femoris muscles of these
animals were cryosectioned and analyzed by
immunofluorescence. Autofluorescence of EBD was not

-20-

detected in fibers from the F1B hamster. In contrast,
EBD was found within groups of fibers in the BIO 14.6
hamster. Strikingly, EBD was excluded from fibers that
had been converted to sarcoglycan-positive by δ-SG
5   adenovirus (expressed for 12 weeks) in the BIO 14.6
hamster. These results demonstrate that sarcolemma
membrane integrity is compromised in the BIO 14.6
hamster, but can be reversed by restoration of the
sarcoglycan complex.

10   Generation of Sqca-null mutant mice.
        In order to design a targeting vector to generate
Sgca-null mice, the murine homologue of the human SGCA
gene was isolated and cloned from a mouse skeletal muscle
library. Murine and human α-sarcoglycan are highly
15   related at the amino acid level and their expression
pattern at the mRNA level is similar (Roberds et al.,
Cell 78, 625-33 (1994); Liu et al., Biochem Biophys Res
Commun 235, 227-35 (1997)). The structural organization
of the gene into ten exons is shared by both species
20   (data not shown, Genbank #AF064081).
        Targeted inactivation of one of the Sgca alleles was
accomplished by replacement of exons 2 and 3 and flanking
intronic sequences with the neomycin resistance gene
(Fig. 1). The targeting construct was designed to create
25   a mutant allele of Sgca representative of certain human
mutations. One third of α-sarcoglycan mutations
characterized to date affect exons 2 and 3 of the SGCA
gene (Carrie et al., J Med Genet 34, 470-5 (1997);
Piccolo et al., Nature Genetics 10, 243-245 (1995)). A
30   total of 1023 colonies surviving G418 and gancyclovir
selection were analyzed by Southern-blot analysis for the
presence of homologous recombination. Using the probe
shown in Fig. 1 (black box), the correctly targeted locus
was shown to contain an EcoR1 fragment of 8.8 kb, while
35   the intact allele had a 5.5 kb band. Two clones yielded
chimeras producing germ-line transmission. Transmission
of the mutant allele followed normal Mendelian

-21-

segregation ratios for an autosomal recessive gene in
mice derived from both clones. Homozygous mutant and
heterozygous newborn pups appeared healthy, showing no
gross developmental abnormalities compared to control

5   littermates.   To determine if the targeting approach
produced a null allele, tissues from homozygous mutants
and heterozygous mice were evaluated and compared them to
wild type mice. Northern blot analysis using a probe
against the full length coding sequence revealed the

10   absence of α-sarcoglycan transcript in *Sgca*-deficient
skeletal and heart tissue from the two independently
derived lines. Reverse transcription-PCR revealed the
presence of a minor transcript in skeletal muscle RNA
resulting from the use of cryptic splicing sites in the

15   neomycin cassette in homozygous mutants and heterozygous
mice. Sequencing of the RT-PCR product revealed that
this mutant transcript encoded exon 1 and a stretch of
516 bp from the inverted neo cassette spliced with exon 4
of the *Sgca* gene. Unexpectedly, this aberrant splicing

20   event inserted 172 amino acids from the non-coding strand
of the neo cassette, maintaining the frame with exon 4.
Translation of the altered transcript would produce a
protein lacking the 91 amino acids encoded by exons 2 and
3, including part of the signal sequence. Using a C-

25   terminal peptide antibody and mAb 20A6 against α-
sarcoglycan this mutant protein could not be detected in
Sgca-deficient skeletal and cardiac tissues by immunoblot
or immunofluorescence analysis.

30   <u>*Sgca*-null mutant mice display a progressive muscular
dystrophy</u>.

          Sgca-null mutant mice did not show any overt signs
of a myopathy, and were in this respect similar to *mdx*
mice.   To examine the progression of the muscular

35   dystrophy in these mutant mice, hematoxylin and eosin
stained frozen sections of the sural triceps and the
diaphragm muscles between the ages of 8 days and 9 months
were evaluated. *Sgca*-null mutant mice started to develop

-22-

a progressive muscular dystrophy at one week of age and,
in contrast to *mdx* mice, showed ongoing muscle necrosis
with increasing age. Pathology characteristic of
muscular dystrophy was observed in every *Sgca*-deficient
5   mouse, but never in control animals. The earliest
changes consisted of widely scattered clusters of
necrotic myocytes or regenerating myocytes with
internally placed nuclei. These clusters increased in
both number and size as the mice increased in age. Based
10   on the evaluation of 200 to 1100 myocytes per muscle, the
number of non-regenerating myocytes with internally
placed nuclei also increased with age. At 8 days,
between 1 and 2.5% of the sural triceps and diaphragm
myocytes already showed central nuclei, respectively.
15   These numbers continuously increased and at 8 and 16
weeks of age more than 70% and as high as 99% of the
Sgca-deficient myocytes contained central nuclei. In
wild type mice, on the other hand, the numbers of
centrally placed nuclei never exceeded 1%.

20   In addition to necrosis, regeneration, and central
nucleation, a broad spectrum of other dystrophic changes
was also noted in *Sgca*-deficient muscle. The most
prominent of these included atrophy, hypertrophy, fiber
splitting, and endomysial fibrosis. In some *Sgca*-
25   deficient mice, 8 weeks of age or older, dystrophic
calcification was noted in association with myocyte
necrosis. Fatty infiltration was present in some of the
muscles from 16 week old mice. A qualitative comparison
of fiber type distribution assessed with ATPase staining
30   as well as staining characteristics with NADH and Gomori
trichrome stains suggested no substantial additional
differences between null mutant and wild type mice at any
age. The homozygotes from both correctly targeted cell
lines demonstrated an identical dystrophic phenotype.

35   <u>Sarcolemmal integrity in α-sarcoglycan-deficient muscle.</u>
          To test whether mutation of the α-sarcoglycan gene
leads to damage of the plasma membrane, *Sgca*-deficient

-23-

mice were intravenously injected with Evans blue dye
(EBD), a normally membrane impermeant molecule. This dye
penetrates into the cytoplasm of fibers with compromised
sarcolemmal integrity. No obvious uptake of the blue

5 tracer into skeletal muscles of heterozygous and control
mice was detected by macroscopic inspection. In
contrast, EBD uptake was consistently observed in
skeletal muscle fibers of the homozygous null mutants.
The extent of EBD accumulation varied among muscles.

10 Areas of blue staining appeared mainly within the
proximal limb muscles and the muscles of the pelvic and
the shoulder girdle. Skeletal muscles which
macroscopically showed dye uptake always revealed red EBD
autofluorescence by fluorescence microscopy analysis.

15 Most of the EBD positive fibers showed intense staining,
whereas in others, the signal was faint. Interestingly,
Evans blue positive fibers were distributed in clusters
throughout the different muscles. Fibers that had taken
up the tracer and were assumed to have pathologic plasma

20 membrane permeability often showed characteristic
features of degeneration and necrosis by H&E staining.
In contrast, dye uptake was not readily visible in
cardiac muscle by macroscopic inspection, and microscopic
analysis revealed no EBD in cardiomyocytes of control or

25 diseased animals.

    Membrane damage in 7 to 10 week old Sgca-deficient
mice was also evaluated by measuring the release of
muscle specific pyruvate kinase (PK) into the circulating
blood (Fig. 2). Age matched wild type and heterozygous

30 mice had normal serum levels of PK activity. Homozygous
mice, on the other hand, exhibited high serum levels of
PK activity similar to that of the mdx mice, indicating
that membrane damage occurred to comparable extents in
Sgca-null mutants as in mdx mice (Fig. 2). Similarly, in

35 older animals (up to 18 weeks of age) no differences in
PK activity between Sgca-null mice and age-matched mdx
mice were found.

-24-

Loss of sarcoglycan and sarcospan expression in Sgca-null
mutant mice.

     Immunofluorescence analysis was performed for each
component of the sarcoglycan complex. In Sgca-deficient
5   mice, α-sarcoglycan protein was absent from the
sarcolemma of skeletal and cardiac muscle fibers. In
addition, there was a concomitant drastic reduction of β-
, γ- and δ-sarcoglycan. Other components of the DGC were
also examined by immunofluorescence microscopy. The
10   laminin α2 chain, α-, and β-dystroglycan were present at
comparable levels with control muscle. However, the
sarcolemmal staining for dystrophin was consistently
patchy and reduced in Sgca-null mutant skeletal muscle
compared to control, although dystrophin staining in
15   heart appeared similar to control. Expression of the
newly identified ε-sarcoglycan (Ettinger et al., J Biol
Chem 272, 32534-8 (1997)) and the 25 kD DGC component
sarcospan (Crosbie, R.H. et al., J Biol Chem 272, 31221-4
(1997)) were also analyzed. In contrast to the other
20   sarcoglycans, skeletal and cardiac muscle staining of ε-
sarcoglycan in Sgca-deficient mice was comparable to
control levels. Interestingly, sarcospan was completely
reduced along with the sarcoglycans at the sarcolemma of
homozygous null mice, indicating a close association
25   between the 25 kD component of the DGC and the
sarcoglycan complex.

Dissociation of the DGC in Sgca-null mutant mice.

     To further examine the expression of DGC components,
immunoblot analysis was performed on isolated membrane
30   preparations from control and homozygous mutant skeletal
muscle. α-sarcoglycan muscle preparations were observed
to be more susceptible to degradation by calcium
dependent cysteine proteases, calpain I and II. Use of
calpain I and II inhibitors allowed the preservation of
35   protein integrity in the membrane preparations from Sgca-
null mice. Coomassie blue staining and staining for

-25-

caveolin-3, the dihydropyridine (DHPR), and the ryanodine
receptor showed that equivalent levels of membrane
proteins were present in control and homozygous mutant
preparations. α-sarcoglycan was not detected in skeletal
5   and cardiac membrane preparations from *Sgca*-null mice.
Heterozygotes expressed control levels of α-sarcoglycan.
β-, γ-, and δ-sarcoglycan were greatly reduced in α-
sarcoglycan-deficient skeletal muscle membranes compared
to control muscle. Dystrophin was slightly reduced in α-
10   sarcoglycan-deficient skeletal muscle in accordance with
the patchy staining observed by immunofluorescence. In
accordance with the dystrophin reduction, levels of the
free radical-producing enzyme neuronal nitric oxide
synthase (nNOS), which is anchored to the sarcolemma by
15   dystrophin, were also reduced. Utrophin was found at
higher levels in membrane-enriched preparations from
homozygous mutant mice compared to control mice. This
observation could be related to the large number of
regenerating fibers in *Sgca*-null mutant muscle, which
20   would be expected to express higher levels of utrophin.
In addition, α- and β-dystroglycan were reduced in α-
sarcoglycan-deficient skeletal muscle membrane
preparations compared to control mice, whereas
immunofluorescence, described above, showed little
25   dystroglycan reduction at the sarcolemma. In the
supernatant from *Sgca*-deficient membrane preparations, α-
dystroglycan was enriched and fully glycosylated, but was
not tightly associated with membranes. Thus, α-
dystroglycan is synthesized correctly, but is not stably
30   anchored to the sarcolemma in the absence of the
sarcoglycan complex. In addition, Western blot confirmed
the immunofluorescence analysis in showing that
dystrophin was no longer tightly held at the skeletal
plasma membrane in the absence of the sarcoglycan
35   complex.

-26-

Abnormal Contractile Properties of *Sgca*-deficient
muscles.

      The body masses of 8 weeks old *Sgca*-null mutant mice
were 25% greater than those of their normal littermates
5   (Fig.3). For the long digital extensor (EDL) muscles of
the *Sgca*-deficient mice, the masses were 55% greater than
muscles of control mice. The absolute forces (P_o), on the
other hand, revealed no differences and the specific P_o in
Sgca-null mice was even 25% lower than in heterozygous
10   littermates. The resistance to stretch of the passive
muscle in homozygous null mutants was 75% greater than in
control animals. In contrast to the changes evident in
the EDL muscles, the *Sgca*-null mutant soleus muscles
responded in a significantly different manner. Compared
15   with soleus muscles in normal littermates, the *Sgca*-null
mutant soleus muscles had a 75% greater mass, a 54%
greater absolute P_o, and no difference in either specific
P_o or resistance to stretch. Based on the direct
measurements of muscle mass (M_m), muscle length (L_m),
20   fiber length (L_f), and maximum isometric tetanic P_o, total
fiber cross-sectional area (CSA) and specific P_o were
calculated. The total fiber CSA (mm^2) was calculated by
dividing the M_m (mg) by the L_f (mm) and then by 1.06
(g/cm^2) to correct for the muscle density (Brooks et al.,
25   *J Physiol (Lond)* **404**, 71-82 (1988)). The force (kN) was
divided by the total fiber CSA (m^2) to obtain an estimate
of the specific force (kN/m^2) of the EDL and soleus
muscles.

      Restoration of the sarcoglycan complex by gene transfer.
30   An adenovirus construct encoding human α-sarcoglycan
was used to test the ability of exogenously provided α-
sarcoglycan cDNA to restore the complex in α-sarcoglycan-
deficient skeletal muscle. An adenovirus construct
encoding human δ-sarcoglycan was used as a control. A
35   recombinant adenovirus encoding either the human α-
sarcoglycan or δ-sarcoglycan cDNA was directly injected
into the quadriceps femoris of 3 week old *Sgca*-null

mutant mice. Five days after injection, muscles were
analyzed for sarcoglycan expression by
immunofluorescence. In the α-sarcoglycan adenovirus-
injected muscle, all components of the complex were
5   restored at the sarcolemma in selected fibers, but the
efficiency of gene replacement after several attempts was
rather low compared to the results we obtained by in
skeletal muscle of the BIO 14.6 cardiomyopathic hamster.
To circumvent a possible immune response against the
10 neoantigen or adenovirus itself, the α-sarcoglycan
adenovirus was injected into the hamstring muscles of 2
day old Sgca-deficient pups. Immunostaining of serial
transverse cryosections of injected muscle after 3 weeks
revealed that high levels of renewed expression of α-
15 sarcoglycan at the sarcolemma were observed. In
addition, α-sarcoglycan positive fibers revealed
expression of the entire sarcoglycan complex, sarcospan
and dystrophin by immunofluorescence microscopy. The
transfection efficiency was greatly improved by injecting
20 at 2 days versus 3 weeks old (with approximately 20 to 50
times more fibers rescued). Immunostaining of the
sarcoglycans was found in up to 70% of fibers, depending
most likely on the delivery and the accuracy of targeting
the hamstrings in the pups. However, in α-sarcoglycan-
25 deficient quadriceps muscles injected with the δ-SG
adenovirus, α-SG was not detected by immunofluorescence,
nor were β-, γ-, and δ-SG. Interestingly, H&E and
immunofluorescence directed at α-SG at 1 month post
injection showed that central nucleation was reduced in
30 injected Sgca-null mutant muscle. In addition, evidence
of α-sarcoglycan expression over a time interval between
five days and 2 months post-injection was observed.

Methods of the Invention.
Animals. Male F1B and BIO 14.6 cardiomyopathic hamsters
35 were obtained from BioBreeders (Fitchburg, MA).

-28-

Antibodies. Rabbit 98 polyclonal antibody against α-SG
has been described previously (Roberds et al.; *J. Biol.
Chem.* 268: 11496 (1993)). Monoclonal antibody 20A6
recognizes α-SG (Piccolo et al.; *Nature Genet.* 5: 1963
5   (1995)). Monoclonal antibody 5B1 was raised against a.a.
89-152 of human β-SG (provided in collaboration with
Louise Anderson). An affinity-purified rabbit antibody
(Rabbit 208) was produced against a C-terminal fusion
protein of rabbit γ-SG. A monoclonal antibody 21B5 was
10   raised against a.a. 167-178 of rabbit γ-SG (provided by
Louise Anderson). An affinity-purified rabbit antibody
(Rabbit 215) was produced against an amino-terminal
peptide of human δ-SG (MMPQEQYTHHRSTMPGAA) (SEQ ID NO:
1). Rabbit 229 recognizes the carboxyl terminus of human
15   δ-SG. Goat 20 serum (Jung et al., *J Biol Chem.* 271,
32321-32329 (1996)) and monoclonal antibody IIH6 (Roberds
et al.; *J. Biol. Chem.* 268: 23739 (1993)) have been
previously described.

Human sarcoglycan expression constructs. Human α-, β-,
20   γ-, δ- and δ$^\Delta$-SG expression constructs were prepared by
PCR (entire constructs) amplification of cDNA using
primers containing appropriate restriction sites for
subcloning into pcDNA3 (Pharmacia). For the δ$^\Delta$-SG
construct, a stop codon was introduced immediately
25   following amino acid 219. Constructs were confirmed by
direct DNA sequence analysis performed by the DNA Core
Facility at the University of Iowa.

Expression of SG expression constructs in CHO cells. CHO
cells were transfected with the empty vector (control) or
30   a mammalian expression vector encoding for α-, β-, γ-, δ-
, and δ$^\Delta$-SG, engineered to include a nine amino acid myc
epitope tag on the intracellular tail. Transfection was
via electroporation (Holt et al., *J. Biol Chem.* 271, 8300
(1996)). Whole cell lysates were prepared and subjected
35   to anti-myc (9E10) immunoblotting.

Recombinant adenovirus. The human δ-SG sequence was
amplified by PCR and subcloned into the pAdCMVpA
adenovirus shuttle vector. The δ-SG construct was then
incorporated into an adenovirus vector and used to infect
5   HEK293 cells by the University of Iowa Gene Transfer
Vector Core. Lysates from the infected cells were
collected and tested for the expression of δ-sarcoglycan
protein using a δ-sarcoglycan-specific polyclonal
antibody. A human α-sarcoglycan adenovirus was made and
10   tested as a control. Recombinant viruses were plaque
purified 3 times, amplified and concentrated using
established methods (Davidson et al., *Exp. Neurology* 125,
258 (1994); Graham et al., *Virology* 52, 456 (1973)).

Plasmid DNA and recombinant adenovirus injections.
15   Hamsters were anesthetized by intraperitoneal injection
of sodium pentobarbital (Nembutal, Abbott Laboratories)
at a calculated dose of 75 mg/kg. The skin overlying the
quadriceps femoris muscle was disinfected and a 1-cm
vertical incision was made. Plasmid DNA (100 µg of
20   sarcoglycan plasmid DNA and 25 µg of β-galactosidase
reporter plasmid DNA) in a total volume of 100 ul in
normal saline (0.9% NaCl w/v) was injected into the
quadriceps femoris muscle (Acsadi et al., *Nature* 352, 815
(1991); Danko et al., *Hum. Mol. Genet.* 2, 2055 (1993);
25   Fritz et al., *Pediatric Research* 37, 693 (1995)). The
incision was closed with 3-4 sutures. For 11 day old
hamsters, the adenovirus was injected directly through
the skin into the hamstring. Hamsters recovered with
continual supervision and were housed post-operatively at
30   the University of Iowa Animal Care Facility. Mice were
housed in BL2 containment with isolator cages. Seven
days to 9 months after injection, hamsters were
euthanized by CO_2 asphyxiation. Injected and uninjected
quadriceps femoris muscle was removed by dissection,
35   embedded in Tissue-Tek O.C.T. compound, and quickly
frozen in liquid N_2-cooled isopentane. For the adenovirus

-30-

studies, 10^9 viral particles in 100 μl of normal saline
were injected into the quadriceps femoris muscle of 3
week-old BIO 14.6 hamsters, and details of the surgery
were as outlined above. All of the above experiments
5   were repeated in at least three animals independently.

Analysis of β-galactosidase activity. Serial 10 mm
cryosections were fixed in 0.5 % glutaraldehyde in PBS
for 15 min at room temperature. After extensive washing
with PBS, sections were treated with 1 mg/ml X-gal in β-
10   galactosidase detection solution (20 mM $K_2Fe(CN)_6$, 20 mM
$K_4Fe(CN)_6$ x $3H_2O$, 2 mM $MgCl_2$) for 2-4 hours. Sections were
counterstained with eosin, mounted with Permount, and
viewed by light microscopy.

Immunofluorescence. Serial 7 μm cryosections were
15   analyzed by immunofluorescence using sarcoglycan-specific
antibodies as described previously (Roberds et al., *J.
Biol. Chem.* **268**, 23739 (1993)). For hematoxylin and
eosin staining, 10 μm sections were stained for 5 min.
each in hematoxylin and then eosin, dehydrated with
20   ethanol and xylenes, mounted with Permount, and examined
by light microscopy. All sections were photographed
under a Zeiss Axioplan fluorescence microscope, a Leitz
Diaplan fluorescence microscope, or a Bio-Rad MRC-600
laser scanning confocal microscope.

25   WGA-Sepharose chromatography and sucrose gradient
fractionation. Quadriceps femoris muscle (1 gram) was
dissected from F1B, BIO 14.6 and BIO 14.6 hamsters
injected with the δ-SG adenovirus, and glycoprotein
preparations were prepared (Iwata et al., *FEBS* **329**, 227
30   (1993)). Briefly, frozen tissue was homogenized in 4 ml
buffer identical or buffer A (Iwata et al., *FEBS* **329**, 227
(1993)) with a polytron for 30 sec. (3X), followed by
Dounce homogenization. Samples were centrifuged in a
Beckman 45Ti rotor at 35,000 rpm for 37 min. The pellet

was resuspended in 4 ml buffer A and the homogenization
steps were repeated. The two supernatants were pooled
and incubated with WGA-Sepharose at 4 °C overnight with
rotation. The resin was washed and proteins were eluted
5   with 0.4 M n-acetyl glucosamine. Eluted proteins were
fractionated by centrifugation speed, length of time
through a 5-30% sucrose gradient. The eluted proteins
and fractions from the sucrose gradient were analyzed by
immunoblotting using specific antibodies and enhanced
10   chemiluminescence.

   In vivo membrane permeability assay. Evans blue dye
injections and microscopic analysis were performed as
described previously (Straub et al., *J. Cell Biol.* 139,
375 (1997)), with some modifications. Evans blue dye was
15   dissolved in phosphate-buffered saline (10 mg/ml) and
sterilized by passage through membrane filters with a
pore size of 0.2 μm. Hamsters were anesthetized by
intraperitoneal injection of sodium pentobarbital
(Nembutal, Abbott Laboratories) at a calculated dose of
20   75 mg/kg. Subsequently 0.25 μl/10 g body weight of the
dye solution was injected intravenously through the front
limb vein. Animals were euthanized 72 hours after
injection by asphyxiation with gaseous CO_2. All dye-
injected hamsters were skinned and inspected for dye
25   uptake in the skeletal muscles, by the appearance of blue
staining. Muscle sections for microscopic Evans blue
detection were incubated in ice cold acetone at -20 °C for
10 min., washed in PBS and mounted with Vectashield
(Vector Laboratories). All sections were examined and
30   photographed under a Zeiss Axioplan fluorescence
microscope.

                    NULL MUTANT EXPERIMENTS

   Isolation of α-sarcoglycan and vector construction. The
35   α-sarcoglycan gene was isolated from a λ FIXII 129/sv

genomic skeletal muscle library by homology screening
using a radiolabeled human α-sarcoglycan cDNA. A 16.2 kb
NotI fragment was characterized by restriction mapping
and limited sequencing (Genbank# AF064081). Two StyI

5   fragments of 0.16 and 0.9 kb carrying exon 1 and a
portion of 5' and 3' contiguous intron were co-ligated
into the XbaI site of pPNT. Orientation of the two
inserted fragments was checked by PCR and sequencing. A
9 kb fragment containing a portion of intron 3, and exon

10   4 to exon 9 was amplified using a high fidelity PCR
reaction (Takara) with intron 3 forward primer from:
CCCCTCGAGCCGTTCCTCAGACTTTTTATTC (SEQ ID NO: 2) and exon 9
reverse primer from: AATGCGGCCGCTCTCCTGTACGAACAT (SEQ ID
NO: 3). The PCR fragment was restriction digested by

15   NotI and XhoI and inserted into a XhoI-NotI cut plasmid.
Correct targeting replaced exon 2 (containing part of the
signal sequence), exon 3, 571 bp of intron 1 and 65 bp of
intron 3 with a neomycin resistance gene in opposite
transcriptional orientation (Fig. 3).

20   Generation of Sgca-deficient mice. The NotI linearized
construct was introduced into $2x10^7$ R_1 ES cells by
electroporation (240V, 500μF; Bio-Rad Gene Pulser). The
ES cells were maintained on feeder layers and passaged
clonally. Targeting fidelity was determined by Southern

25   analysis, which was performed using the probe shown
(black box) in Fig 3. Cells from three correctly
targeted clones were microinjected into C57BL/6J
blastocysts and transferred into pseudopregnant
recipients. Following germ-line transmission, genotypes

30   were determined by PCR on DNA from tail biopsies. Primer
sites are shown in Fig. 3 as horizontal arrows. The
following primers and PCR conditions were used: C-KO2 in
intron 1: CAGGGCTGGGAGCTGGGTTCTG (SEQ ID NO: 4); MFD1 in
intron3 (deleted in the null allele): CCCAGGGCCTTGATGCCT

35   (SEQ ID NO: 5); and NEOTR: GCTATCAGGACATAGCGTTGGCTA (SEQ
ID NO: 6): first denaturation at 94°C for 5 min; followed
by 30 cycles of 1 min, 94°C, 1 min 64°C, 2 min 30 sec at

-33-

72°C, and 7 min last extension at 72°C. Wild type and
null alleles corresponded to PCR fragments of 1061 bp and
618 bp respectively.

Northern-blot analysis. Total RNA from control,
5   heterozygous, and homozygous null mutant skeletal and
cardiac tissues was extracted using RNAzol (Tel-Test)
according to manufacturer specifications. 30 µg of total
RNA was run on a 1.25% agarose gel containing 5%
formaldehyde and transferred to Hybond N membrane
10   (Amersham). RNA was cross-linked to the membrane using a
Stratagene UV crosslinker. Membranes were then
prehybridized and hybridized using standard methods.
Washes were carried out at 65 °C in 1x SSC/1% SDS
initially, then 0.1x SSC/0.1% SDS. Blots were exposed
15   for autoradiography.

Evans blue dye injection and microscopic evaluation.
Evans blue (Sigma Chemical Co., St. Louis, MO) was
dissolved in PBS (10 mg/ml) and sterilized by passage
through membrane filters with a pore size of 0.2 µm.
20   Mice were injected intravenously with 0.25 µl/10 g body
weight of the dye solution through the tail vein.
Animals were sacrificed 6 hours after injection by
cervical dislocation. During the time period between
injection and cervical dislocation, animals were kept in
25   standard laboratory cages. All mice were skinned and
inspected for dye uptake in the skeletal muscles,
indicated by blue coloration. Muscle sections for
microscopic Evans blue detection were incubated in ice
cold acetone at -20°C for 10 min, and after a rinse with
30   PBS, sections were mounted with Vectashield mounting
medium (Vector). Sections were observed under a Zeiss
Axioplan fluorescence microscope (Carl Zeiss, Inc.,
Thornwood, NY) or a MRC-600 laser scanning confocal
microscope (Bio Rad Laboratories, Hercules, CA).

<u>Pyruvate Kinase Assay</u>. Activities of muscle specific
pyruvate kinase isozyme found in the blood serum were
measured as previously documented (Edwards et al., *Clin
Chem.* **27**, 906-909 (1981)). Blood was collected from the
5   ·retroorbital sinus of 2 to 18 week old mice and the serum
was stored at -80°C prior to measurements.

<u>Antibodies</u>. Monoclonal antibodies IIH6 against α-
dystroglycan (Ervasti et al., *Cell* **66**, 1121-1131 (1991))
and 8D5 against β-dystroglycan (Lim et al., *Nature*
10   *Genetics* **11**, 257-265 (1995)) were previously
characterized. Monoclonal antibodies 20A6 against α-
sarcoglycan, 5B1 against β-sarcoglycan, and 21B5 against
γ-sarcoglycan were generated in collaboration with Louise
Anderson. The monoclonal antibody against caveolin-3 was
15   purchased from Transduction Laboratories. Rabbit
polyclonal antibodies against α-sarcoglycan (Roberds et
al., *J Biol Chem* **268**, 23739-23742 (1993)), dystrophin and
utrophin (Ohlendieck et al., *Neuron* **7**, 499-508 (1991)),
nNOS (Crosbie et al., *Hum Mol Genet* **7**, 823-9 (1998)), α_1-
20   DHPR (Ohlendieck et al., *J Cell Biol* **112**, 135-148
(1991)), and the laminin α2 chain (Allamand et al., *Hum
Mol Genet* **6**, 747-52 (1997)) were described previously.
Two affinity-purified rabbit antibodies (Rabbit 208 and
215) were produced against a C-terminal fusion protein of
25   γ-sarcoglycan, and against an N-terminal peptide
(MMPQEQYTHHRSTMPGAA) (SEQ ID NO: 1) of δ-sarcoglycan
affinity-purified rabbit antibody respectively. An
affinity-purified goat antibody (Goat 26) was produced
against a N-terminal fusion protein of β-sarcoglycan
30   containing amino acids 1 to 65. Polyclonal antibodies
against α-dystroglycan fusion protein D (Ibraghimov-
Beskrovnaya et al., *Nature* **355**, 696-702 (1992)) were
affinity-purified from goat 20. An affinity-purified
rabbit antibody (Rabbit 235) was produced against a C-
35   terminal fusion protein of sarcospan
(CFVMWKHRYQVFYVGVGLRSLMASDGQLPKA) (SEQ ID NO: 7). Two

polyclonal antibodies against ε-sarcoglycan were used.
One was previously characterized (Ettinger et al., *J Biol
Chem* 272, 32534-8 (1997)) and one was obtained from
affinity purified rabbit antibody (Rabbit #232) against a

5   C-terminal peptide of ε-sarcoglycan (PHQTQIPQQQTTGKWYP)
(SEQ ID NO: 8).

Immunofluorescence Analysis. For immunofluorescence, 7
μm transverse cryosections were prepared from control and
Sgca-null mutant skeletal and cardiac muscle. All

10   procedures were performed at room temperature. Sections
were blocked with 5% BSA in PBS for 1 hr., and then
incubated with the primary antibodies for 90 min. After
washing with 1% BSA/PBS, sections were incubated with
Cy3-conjugated secondary antibodies (1:250) for 1 hr.,

15   washed with 1% BSA/PBS. After a rinse with PBS, sections
were mounted with Vectashield mounting medium (Vector)
and observed under a Zeiss Axioplan fluorescence
microscope.

Contractile Properties. For the measurement of

20   contractile properties of EDL or soleus muscles of
control or *Sgca*-deficient mice *in vitro*, mice were
anesthetized with sodium pentobarbital (30 to 50 mg/kg
for control or *Sgca*-deficient mice, respectively).
Supplemental doses of the anesthetic were given as

25   required to maintain a level of anesthesia sufficient
that mice did not respond to tactile stimuli. Prior to
the measurement of the contractile properties, each mouse
was weighed. Contractile properties were measured on 28
muscles, 6 EDL and 6 soleus muscles from control and 8

30   EDL and 8 soleus muscles from *Sgca*-null mutant mice. The
muscles were isolated and removed carefully from the
anesthetized mice and immersed in an oxygenated (95% O_2
and 5% CO_2) bath containing a buffered mammalian Ringer's
solution (pH 7.4), which included curare. The solution

35   was maintained at 25°C. The tendons were tied securely
to a servomotor and a force transducer. The muscles were

-36-

stimulated directly by the current flow between two large
platinum electrodes (Brooks et al., *J Physiol (Lond)* 404,
71-82 (1988)). The voltage of the stimulator was set to
provide maximum twitch force and the L_m was set at optimum

5   length (L_o) for force development. With the muscle at L_o,
the frequency of stimulation was increased until force
plateaued at P_o. Following the measurement of P_o, the
length of the muscle was set at 90% of L_f and with the
muscle passive the muscle was stretched to 110% of L_f at 1

10 L_f/s and then returned at the same velocity to 90% of L_f.
The peak force at the end of the stretch was used as a
measure of the resistance to stretch of the passive
muscle. The muscle was removed from the bath, blotted
and weighed to obtain the M_m. Each percentiled data set

15 was analyzed by a two way analysis of variance (ANOVA) in
a general linear model algorithm appropriate for unequal
sample sizes (Statistical Analysis System, Gary, NC). In
circumstances where the overall F-ratio for the ANOVA was
significant, the differences between individual group

20 means were determined by post hoc pairwise t-comparisons
of least square means with appropriate correction of the
significance level to account for multiple comparisons.
Significant differences between data on control and *Sgca*-
deficient mice are indicated in Fig. 5 by asterisks and

25 the differences between the EDL and the soleus muscles
are indicated with a sword. Significance was set a
priori at $p < 0.05$.

Recombinant Adenovirus Injections. The human α-
sarcoglycan cDNA sequence was subcloned into the pAdRSVpA

30 adenovirus vector through standard methods of homologous
recombination with Ad5 backbone dl309 by the University
of Iowa Gene Transfer Vector Core. Lysates from the
infected cells were collected and tested for the
expression of α-sarcoglycan using a polyclonal antibody.

35 Recombinant viruses were plaque purified 3 times,
amplified and concentrated using established methods
(Davidson et al., *Exp Neurol* 125, 258-67 (1994); Graham

et al., *Virology* 54, 536-9 (1973)). Recombinant
adenovirus injections were performed as previously
described (Holt et al., *Mol Cell* 1, 841-848 (1998)). For
2 day old *Sgca*-null mutant mice, the adenovirus was

5   injected directly through the skin into the hamstring.
Three week old mice were anesthetized by intraperitoneal
injection of sodium pentobarbital (Nembutal, Abbott
Laboratories) at a calculated dose of 50 mg/kg. A human
δ-sarcoglycan adenovirus was used as control.

10   Serial transverse cryosections of injected muscle
after 3 weeks, were stained with the following
antibodies: α-sarcoglycan (α-SG), β-sarcoglycan (β-SG),
γ-sarcoglycan (γ-SG), and δ-sarcoglycan (δ-SG),
dystrophin (DYS) and sarcospan (SPN).

15   Reduction of central nucleation in fibers expressing
recombinant α-sarcoglycan adenovirus was detected in two
day old homozygous mutant mice that were injected as
described above. Quadriceps muscles were analyzed 1
month post-injection by H&E and immunofluorescence using

20   a polyclonal antibody against α-sarcoglycan (α-SG), 1
month post-injection.

Immunoblot analysis of membrane enriched preparations.
Crude surface membranes from skeletal and cardiac muscle
were prepared as described previously (Ohlendieck et al.,

25   *Journal of Cell Biology* 112, 135-148 (1991)). Membranes
were resolved by SDS-polyacrylamide gel electrophoresis
(SDS-PAGE) on 3-12% linear gradient gel and transferred
to nitrocellulose membrane. Immunoblot staining was
performed as previously described (Ohlendieck et al.,

30   *Journal of Cell Biology* 112, 135-148 (1991)).

GenBank Accession Numbers. The GenBank accession number
for the mouse genomic sequence of α-SG gene is:
AF064081.

CLAIMS

1. A method for treating mammalian sarcoglycan-
   deficient limb-girdle muscular dystrophy in an
   individual, comprising the steps:
   a) determining the sarcoglycan species containing
      a mutation which is responsible for the
      sarcoglycan-deficient limb-girdle muscular
      dystrophy in the individual;
   b) providing an expression vector containing a
      wild-type form of the sarcoglycan species; and
   c) introducing the expression vector into muscle
      tissue under conditions appropriate for
      expression of the wild-type form of the
      sarcoglycan species.

2. The method of Claim 1 wherein the sarcoglycan
   species is selected from the group consisting of
   alpha, beta, gamma, or delta sarcoglycan.

3. The method of Claim 2 wherein the sarcoglycan
   species is alpha sarcoglycan.

4. The method of Claim 2 wherein the sarcoglycan
   species is delta sarcoglycan.

5. The method of Claim 2 wherein the determination of
   the sarcoglycan species containing the mutation
   responsible for the sarcoglycan-deficient limb-
   girdle muscular dystrophy is based on a detected
   absence or substantial reduction in the wild-type
   sarcoglycan species, or the mRNA which encodes the
   wild-type sarcoglycan species.

6. The method of Claim 2 wherein the determination
   of the sarcoglycan species containing the
   mutation responsible for the sarcoglycan-
   deficient limb-girdle muscular dystrophy is

made by hybridization methods using a nucleic
acid probe specific for a predetermined
mutation in the sarcoglycan species.

7.   The method of Claim 2 wherein the determination of
the sarcoglycan species containing the mutation
responsible for the sarcoglycan-deficient limb-
girdle muscular dystrophy is made by base pair
sequencing of the sarcoglycan gene exons, and
comparing the determined sequence to wild type
sarcoglycan gene exons.

8.   A method for determining a specific defective
sarcoglycan species in diseased tissue of a patient,
comprising the steps:
a)   performing a biopsy of the diseased tissue;
b)   generating a muscle cell culture from the
biopsied tissue of step a) under conditions
appropriate for the formation of myotubes;
c)   providing expression vectors that encode each
of the sarcoglycan species;
d)   independently introducing the expression
vectors of step c) to the muscle cells of step
b) under conditions appropriate for expression;
and
d)   determining which of the introduced sarcoglycan
species of step d) restores the dystrophin-
glycoprotein complex to the cells, restoration
being indicative of the sarcoglycan species
defective in the patient.

9.   The method of Claim 1 wherein the expression vector
is selected from the group consisting of an
adenovirus expression vector, a gutted adenovirus
expression vector and an adeno-associated expression
vector.

10. The method of Claim 1 where the expression vector
     contains a muscle tissue-specific promoter.

11. The method of Claim 1 wherein the expression vector
     is introduced by intramuscular injection.

12. A mouse, and cells derived therefrom, homozygous for
     a disrupted α-sarcoglycan gene, the disruption in
     said gene having been introduced into the mouse or
     an ancestor of the mouse at an embryonic stage,
     wherein the disruption prevents the synthesis of
     functional α-sarcoglycan in cells of the mouse and
     results in the mutant mouse having no detectable
     sarcospan, β-, γ-, and δ-sarcoglycan, and reduced α-
     dystroglycan in the sarcolemma of skeletal and
     cardiac muscles, and a reduction of dystrophin in
     skeletal muscle compared to the amounts of said
     components in a mouse lacking disrupted α-
     sarcoglycan genes.

13. The mouse, and cells derived therefrom, of Claim 12
     wherein the disruption comprises a deletion of a
     region of 902 base pairs, that includes exon 2, exon
     3, 571 base pairs of intron 1, and 65 base pairs of
     intron 3, and replacement with a PGK-neomycin
     cassette as a marker for neomycin resistance.

14. The mouse, and cells derived therefrom, of Claim 13
     wherein said deletion results from the introduction,
     into embryonic stem cells, of a DNA construct
     comprising:
     a)   exons 1, 4-6 of the α-sarcoglycan gene; and
     b)   a neomycin resistance gene inserted between
          intron 1 and intron 3 of the α-sarcoglycan
          gene, said neomycin resistance gene being in
          the opposite transcriptional orientation as
          exons 1, 4-6 of the α-sarcoglycan gene, and

wherein said construct lacks exons 2-3 of the
α-sarcoglycan gene.

15. A mouse, and cells derived therefrom, homozygous for
a disrupted δ-sarcoglycan gene, the disruption in
said gene having been introduced into the mouse or
an ancestor of the mouse at an embryonic stage,
wherein the disruption prevents the synthesis of
functional δ-sarcoglycan in cells of the mouse and
results in the mutant mouse having a reduced amount
of sarcospan, α-, β-, and γ-sarcoglycan in the
sarcolemma of skeletal and cardiac muscles, and a
reduction of β-sarcoglycan and sarcospan in smooth
muscle compared to the amounts of said components in
a mouse lacking disrupted δ-sarcoglycan genes.

16. The mouse, and cells derived therefrom, of Claim 15
wherein the disruption comprises a deletion of a
region of 2992 base pairs, including 2277 base pairs
of intron 1, the entire exon 2, and 576 base pairs
of intron 2, and replacement of the deleted region
with a PGK-neomycin cassette as a marker for
neomycin resistance.

17. The mouse, and cells derived therefrom, of Claim 16
wherein said deletion results from the introduction,
into embryonic stem cells, of a DNA construct
comprising:
a)   5 kb of intron 1 and 5 kb of intron 2 of the δ-
sarcoglycan gene; and
b)   a neomycin resistance gene inserted between
intron 1 and intron 2 of the δ-sarcoglycan
gene, said neomycin resistance gene being in
the opposite transcriptional orientation as the
δ-sarcoglycan exons replaced, wherein said
construct lacks exon 2 of the δ-sarcoglycan
gene.

18.   A method for identifying therapeutic agents for
      the treatment of sarcoglycan-deficient limb
      girdle muscular dystrophy, comprising:
      a)   providing a mouse homozygous for a
            disrupted sarcoglycan gene;
      b)   administering a candidate therapeutic
            agent to the mouse of step a); and
      c)   assaying for therapeutic effects on
            the mouse of step a), the
            determination of therapeutic effects
            being a reduction or reversal in
            disease progression.

19.   The method of Claim 18 wherein the therapeutic
      agent is a gene.

20.   The method of Claim 18 wherein the therapeutic
      agent is a compound.

21.   The method of Claim 18 wherein the disrupted
      sarcoglycan gene is a member of the group α-,
      β-, γ-, and δ-sarcoglycan.

22.   The method of Claim 21 wherein the disrupted
      sarcoglycan gene is α-sarcoglycan.

23.   The method of Claim 21 wherein the disrupted
      sarcoglycan gene is δ-sarcoglycan.

24.   The method of identifying therapeutic agents in
      the treatment of sarcoglycan deficient limb
      girdle muscular dystrophy, comprising:
      a)   providing a mammalian cell in culture
            homozygous for a disrupted
            sarcoglycan gene;

b) administering a candidate therapeutic
    agent to the mammalian cell of step
    a); and

c) assaying for therapeutic effects on
    the mammalian cell of step a) by the
    candidate therapeutic agent of step
    b), the determination of therapeutic
    effects being the complete or partial
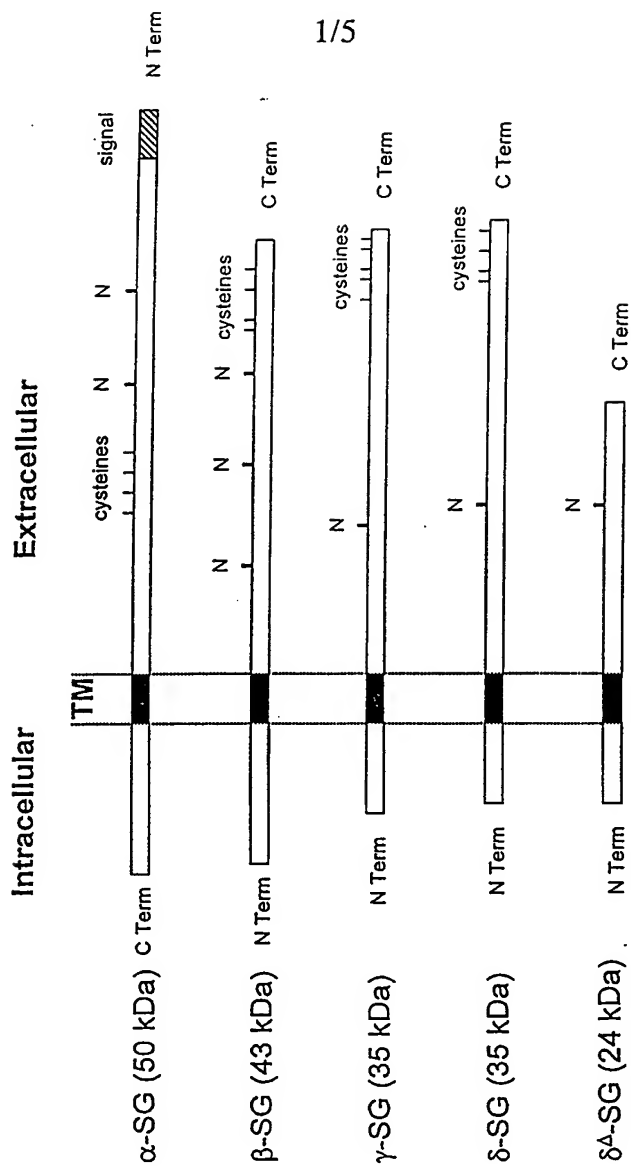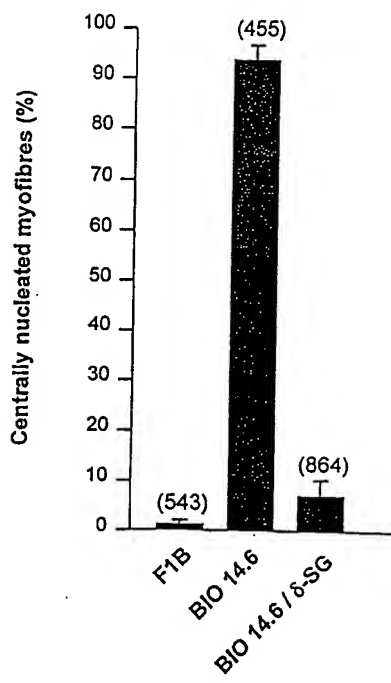   restoration of the dystroglycan
    complex.

FIG. 1
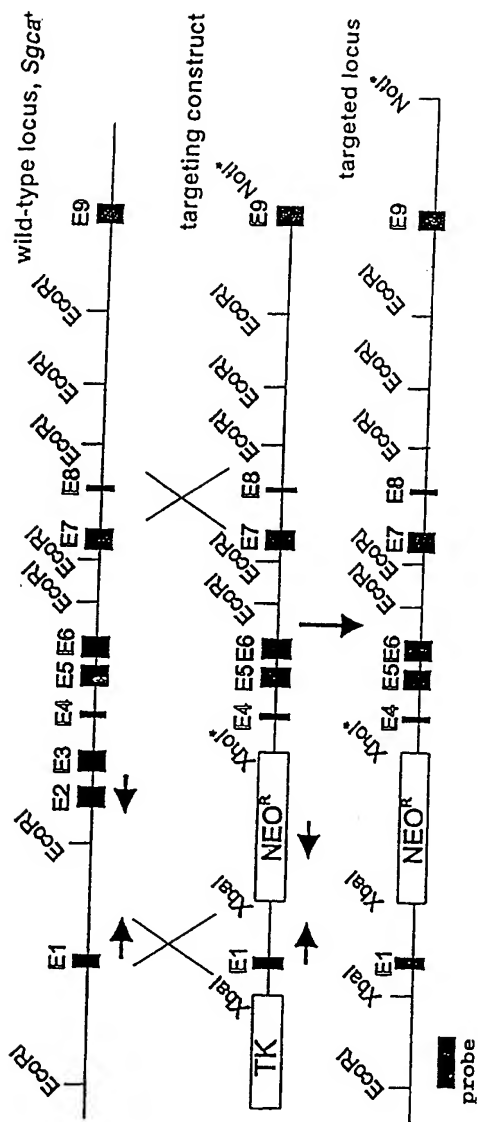
2/5

FIG. 2

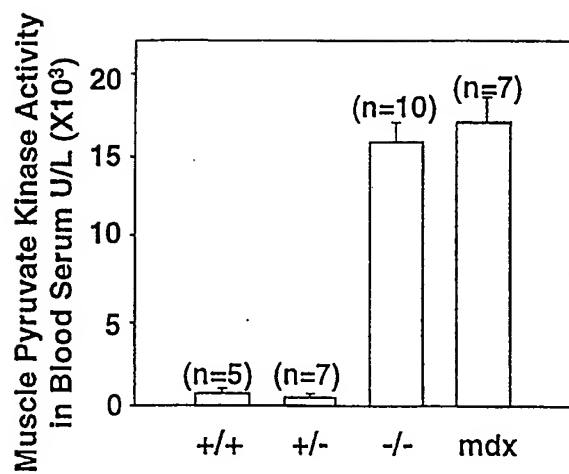FIG. 3

FIG. 4

5/5

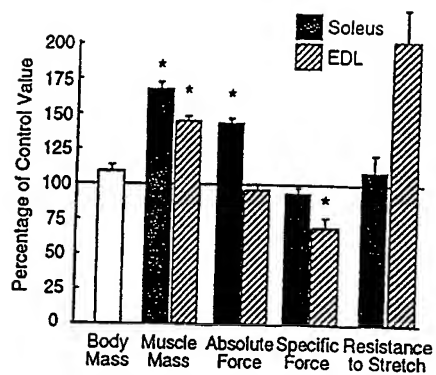
FIG. 5

SEQUENCE LISTING

<110> University of Iowa Research Foundation

<120> GENE REPLACEMENT THERAPY FOR MUSCULAR DYSTROPHY

<130> UIRF-2003WO

<140>
<141>

<150> US 09/164,664
<151> 1998-10-01

<160> 8

<170> PatentIn Ver. 2.0

<210> 1
<211> 18
<212> PRT
<213> Homo sapiens

<400> 1
Met Met Pro Gln Glu Gln Tyr Thr His His Arg Ser Thr Met Pro Gly
 1   5   10   15

Ala Ala

<210> 2
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR primer for
     analysis

<400> 2
cccctcgagc cgttcctcag actttttatt c   31

<210> 3
<211> 27
<212> DNA
<213> Artificial Sequence

```
<220>
<223> Description of Artificial Sequence:PCR primer for
      analysis

<400> 3
aatgcggccg ctctcctgta cgaacat                                    27

<210> 4
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR primer for
      analysis

<400> 4
cagggctggg agctgggttc tg                                          22

<210> 5
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR primer for
      analysis

<400> 5
cccagggcct tgatgcct                                               18

<210> 6
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR primer for
      analysis

<400> 6
gctatcagga catagcgttg gcta                                        24

<210> 7
<211> 31
<212> PRT
<213> Artificial Sequence
```

<220>
<223> Description of Artificial Sequence: polypeptide
     fragment for use as antigen

<400> 7
Cys Phe Val Met Trp Lys His Arg Tyr Gln Val Phe Tyr Val Gly Val
 1   5   10   15

Gly Leu Arg Ser Leu Met Ala Ser Asp Gly Gln Leu Pro Lys Ala
             20   25   30

<210> 8
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:polypeptide
     fragment for use as antigen

<400> 8
Pro His Gln Thr Gln Ile Pro Gln Gln Gln Thr Thr Gly Lys Trp Tyr
 1   5   10   15

Pro